invitrogen™

pUni/V5-His A, B, and C Echo™ Cloning System

Construction of a donor vector for recombination with an Echo™-adapted acceptor expression vector

Catalog no. ET003-XX

Version E
11 November 2010
25-0373

User Manual

Table of Contents

Table of Contents iii
Kit Contents and Storage iv
Product Qualification vi
Accessory Products vii
Purchaser Notification viii

Introduction 1

Overview 1

Methods 5

Cloning into pUni/V5-His A, B, and C 5
Transformation with One Shot® *E. coli* 10
Designing Your Own Echo™-Adapted Expression Vector 13

Appendix 15

Recipes 15
Map of pUni/V5-His A, B, and C Vector 16
Features of pUni/V5-His A, B, and C Vector 17
Technical Service 18
References 19

Kit Contents and Storage

Types of Kits Available

pUni/V5-His A, B, and C Echo™ Cloning System Donor Vector Module is available in two formats. Refer to the table below for the kit you ordered.

Kit	Catalog no.
Echo™ Cloning System Donor Vector Module with One Shot® PIR1 Chemically Competent *E. coli*	ET003-10
Echo™ Cloning System Donor Vector Module with One Shot® PIR2 Chemically Competent *E. coli*	ET003-11

Shipping and Storage

The Echo™ Cloning System Donor Vector module is shipped at room temperature. Each kit contains a vector kit (Box 1) and One Shot® PIR1 or PIR2 Chemically Competent *E. coli* (Box 2).

Reagents	Storage
pUni/V5-His A, B, and C vector kit	Room temperature
One Shot® PIR1 or PIR2 Chemically Competent *E. coli*	-80°C

Contents

The following items are supplied with the Echo™ Cloning System Donor Vector kit.

Item	Amount
pUni/V5-His A, B, and C	20 µg each, lyophilized in TE, pH 8.0
pUni Forward Sequencing Primer	2 µg, lyophilized in TE, pH 8.0
pUni Reverse Sequencing Primer	2 µg, lyophilized in TE, pH 8.0

One Shot® Reagents

The table below describes the items included in each One Shot® Chemically Competent *E. coli* Kit. **Store at -80°C.**

Item	Composition	Amount
SOC Medium (may be stored at +4°C or room temperature)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
PIR1 or PIR2	Chemically Competent *E. coli*	11 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0	50 µl

Continued on next page

Sequence of Primers

The table below lists the sequence and pmoles supplied of the sequencing primers included in this kit.

Primer	Sequence	pmoles Supplied
pUni Forward Sequencing Primer	5′-CTATCAACAGGTTGAACTG-3′	345
pUni Reverse Sequencing Primer	5′-CAGTCGAGGCTGATAGCGAGCT-3′	295

Genotype of *E. coli* Strains

PIR1: You may use this strain for cloning and maintenance of your donor vector construct. This strain contains a mutant allele of the *pir* gene, which maintains the donor vector construct at ~250 copies per cell.

F$^-$ *Δlac169 rpoS(Am) robA1 creC510 hsdR514 endA recA1 uidA(ΔMlu I)::pir-116*

PIR2: This strain is recommended for maintaining constructs that express toxic genes or for libraries. Use this strain for cloning and maintenance of your donor vector construct. This strain contains the wild-type *pir* gene for maintenance of the donor vector construct at ~15 copies per cell.

F$^-$ *Δlac169 rpoS(Am) robA1 creC510 hsdR514 endA recA1 uidA(ΔMlu I)::pir*

PIR1 and PIR2 strains are derived from K-12 strain.

Pir Gene

The *pir* gene encodes the replication protein π that is required to replicate and maintain plasmids containing the R6Kγ origin such as pUni/V5-His A, B, and C.

Product Qualification

Introduction

Invitrogen qualifies the Echo™ Cloning System Donor Vector kit as described below.

Restriction Digest

Supercoiled pUni/V5-His is qualified by restriction enzyme digestion. Restriction digests must demonstrate the correct banding patterns when electrophoresed on an agarose gel.

Vector	Restriction Enzyme	Expected Results (bp)
pUni/V5-His A	*Pvu* I linearizes	2290
	*Bam*H I linearizes	2290
	Bgl II and *Bgl* I	1498, 792
pUni/V5-His B	*Sal* I linearizes	2297
	*Bam*H I linearizes	2297
	Bgl II and *Bgl* I	1485, 812
pUni/V5-His C	*Nru* I linearizes	2295
	*Bam*H I linearizes	2295
	Bgl II and *Bgl* I	1483, 812

Primers

Both primers have been lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

One Shot® PIR1 and PIR2 Competent *E. coli*

To qualify PIR1 and PIR2 cells:

1. 50 µl of competent cells are transformed with 10 pg of supercoiled pUC19 plasmid. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be:
   - PIR1: > 1 x 10^9 cfu/µg DNA
   - PIR2: > 1 x 10^8 cfu/µg DNA
2. Transformation efficiency is also confirmed with supercoiled pUni/V5-His (10 pg). Transformed cultures are plated on LB plates containing 50 µg/ml kanamycin and the transformation efficiency calculated.
3. Untransformed cells are plated on:LB plates containing 100 µg/ml ampicillin, 25 µg/ml streptomycin, 50 µg/ml kanamycin, 15 µg/ml tetracycline, or 15 µg/ml chloramphenicol to verify the absence of antibiotic- resistant contamination.
4. To verify the absence of phage contamination, 0.5-1 ml of competent cells are added to LB top agar and poured onto LB plates. After overnight incubation, no plaques should be detected.

Accessory Products

Additional Products

The table below lists additional products that may be useful for creating and characterizing recombinant fusion plasmids.

Product	Amount	Catalog no.
One Shot® PIR1 Chemically Competent *E. coli*	11 x 50 µl	C1010-10
One Shot® PIR2 Chemically Competent *E. coli*	11 x 50 µl	C1111-10
Anti-V5 Antibody	50 µl*	R960-25
Anti-V5-HRP Antibody	50 µl*	R961-25
Anti-His (C-term) Antibody	50 µl*	R930-25
Anti-His (C-term)-HRP Antibody	50 µl*	R931-25

*Quantity supplied is sufficient for 25 western blots.

Echo™-Adapted Acceptor Vectors

Invitrogen has a variety of Echo™-adapted acceptor vectors for expression of your gene of interest in bacterial, yeast, insect, and mammalian host systems. These include acceptor vectors for inducible or constitutive expression. We are constantly adding to our collection of Echo™-adapted acceptor vectors. For more information, visit our Web site (www.invitrogen.com) or contact Technical Service (see page 18).

Purchaser Notification

Limited Use Label License No. 22: Vectors and Clones encoding Histidine Hexamer

This product is licensed under U.S. Patent Nos. 5,284,933 and 5,310,663 and foreign equivalents from Hoffmann-LaRoche, Inc., Nutley, NJ and/or Hoffmann-LaRoche Ltd., Basel, Switzerland and is provided only for use in research. Information about licenses for commercial use is available from QIAGEN GmbH, Max-Volmer-Str. 4, D-40724 Hilden, Germany.

Limited Use Label License No. 119: Echo™ Cloning Products

No license is conveyed to use this product with any recombination sites other than those purchased from Life Technologies Corporation or its authorized distributor. The buyer cannot modify the recombination sequence(s) contained in this product for any purpose.

Limited Use Label License No. 358: Research Use Only

The purchase of this product conveys to the purchaser the limited, non-transferable right to use the purchased amount of the product only to perform internal research for the sole benefit of the purchaser. No right to resell this product or any of its components is conveyed expressly, by implication, or by estoppel. This product is for internal research purposes only and is not for use in commercial applications of any kind, including, without limitation, quality control and commercial services such as reporting the results of purchaser's activities for a fee or other form of consideration. For information on obtaining additional rights, please contact outlicensing@lifetech.com or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

Introduction

Overview

Introduction

The Echo™ Cloning System is based on the univector plasmid-fusion system (UPS) described by Elledge and coworkers, which quickly and easily recombines a gene of interest into a series of recipient (acceptor) vectors (Liu *et al.*, 1998; Liu *et al.*, 1999). The donor vector, pUni/V5-His A, B, and C, is used for restriction enzyme-mediated cloning of a gene of interest and subsequent recombination with an Echo™-adapted acceptor vector.

pUni/V5-His A, B, and C

pUni/V5-His A, B, and C is a set of donor vectors (approximately 2.3 kb each) containing the following features.

- *loxP* site placed adjacent to the 5′ end of the multiple cloning site for site-specific recombination with Echo™-adapted acceptor vectors
- Multiple cloning site for insertion of the gene of interest
- An optional C-terminal tag encoding the V5 epitope for detection and a 6xHis tag for purification
- Three reading frames to facilitate in-frame cloning with the C-terminal tag
- Bacterial and eukaryotic transcription termination sequences for efficient processing in the host of choice
- R6Kγ origin for propagation of the plasmid in strains that express the essential replication protein π (encoded by the *pir* gene)
- *neo* gene for resistance to kanamycin and selection in *E. coli*

For a map and more information on each feature, see pages 16-17.

Site-Specific Recombination

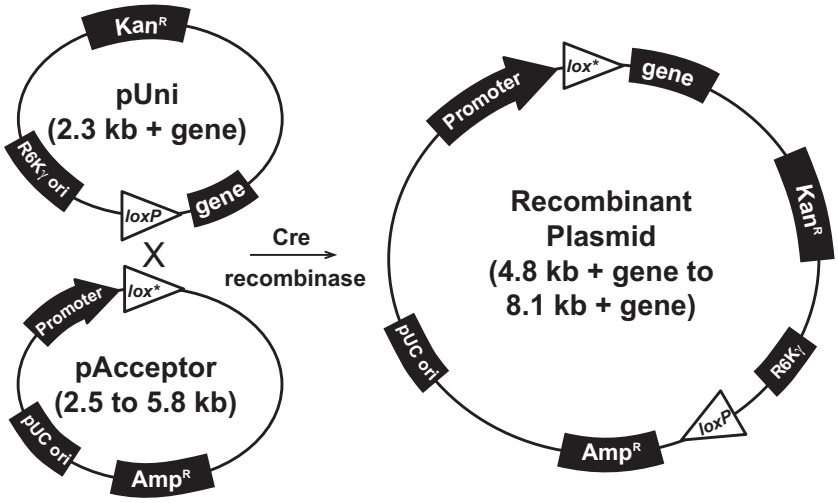
The Echo™ system utilizes the *cre-lox* site-specific recombination system of bacteriophage P1 (Abremski *et al.*, 1983; Sternberg *et al.*, 1981). The product of the *cre* gene is a site-specific recombinase that catalyzes recombination between two 34 bp *loxP* sites to resolve P1 dimers generated by replication of circular lysogens.

Continued on next page

Overview, Continued

Plasmid Fusion

The donor vector and the Echo™-adapted acceptor vector each contain a single *lox* site. The donor vector contains a *loxP* site while the acceptor vector (2.5 to 5.8 kb) contains either a *loxP* or *loxH* site (for more information on *loxH*, see below). Acceptor vectors contain the appropriate transcription regulatory sequences that will control expression of the gene of interest in the desired host system. These acceptor vectors may also carry translation initiation and additional coding sequences for generation of fusion proteins. The unique *loxP* or *loxH* site is located downstream of these sequences. By mixing the donor vector containing the gene of interest with the desired acceptor vector in the presence of Cre recombinase, a plasmid fusion is created that expresses the gene of interest in the appropriate host. The size of the fusion plasmid can range from 4.8 to 8.1 kb (**without** the gene of interest).



*lox** = *loxP* or *loxH* depending on acceptor vector

loxP or loxH

The sequence of the *loxP* site is shown below. It consists of a 34 bp sequence containing a 13 bp inverted repeat separated by an 8 bp spacer region (Hoess *et al.*, 1982). The inverted repeat (underlined) may form a stem and loop structure that may reduce expression of the gene of interest in some cases. A variation of the *loxP* site (*loxH*) was created to eliminate the formation of a stem and loop structure and potentially improve transcription. Mutated bases are shown in boldface.

- *loxP*: ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA T
- *loxH*: AT**T** AC**C** TC**A** TAT AGC ATA CAT TAT ACG AAG TTA T

Note: Data from our experiments do not show any difference in expression levels from vectors having the *loxP* or the *loxH* site.

Continued on next page

Overview, Continued

Cre Recombinase

A simple way to think of Cre recombinase (MW = 35 kDa) is that it is a combination between a restriction enzyme and ligase. It binds to specific sequences (*loxP* or *loxH* sites) on the DNA, brings together the target sites, cleaves them, and covalently attaches to the DNA. Recombination occurs following two pairs of strand exchanges and ligation of the DNAs in a novel (recombinant) form.

Note: Cre catalyzes recombination between two *loxP* sites or between a *loxP* and a *loxH* site; it does not catalyze recombination between two *loxH* sites.

A nucleophilic hydroxylated tyrosine initiates the DNA cleavage event by attack on a specific phosphodiester bond followed by the covalent attachment of the recombinase to the target sequence through a phosphoamino acid bond (Abremski and Hoess, 1992; Argos *et al.*, 1986). The reaction does not require any host factors or ATP, but does require Mg^{2+} or spermidine for activity (Abremski *et al.*, 1983). *In vitro* recombination between two supercoiled substrates, each containing a *loxP* or *loxH* site, results in a supercoiled dimer. The extent of the reaction is 10-20% under optimal conditions (Abremski and Hoess, 1984; Abremski *et al.*, 1983).

Acceptor Vectors

Various Echo™-adapted acceptor vectors are available and are provided with their own manuals. Briefly, each vector contains the following features.

- A *loxP* or a *loxH* site for plasmid fusion
- A specific promoter residing upstream of the *loxP* or *loxH* site for expression in the appropriate host
- A resistance marker for selection in *E. coli* and/or for creation of stable cell lines in eukaryotes
- The pUC origin for high copy replication and maintenance in most *E. coli* strains

Echo™-adapted acceptor vectors may also contain sequences for propagation, selection, and maintenance for organisms other than *E. coli*. For more information on available acceptor vectors, visit our Web site (www.invitrogen.com) or call Technical Service (see page 18). If you wish to Echo™-adapt your own vector, see pages 13-14.

Transformation

Once you complete ligation of your gene of interest into pUni/V5-His A, B, or C, transform the ligation reaction into PIR1 or PIR2 cells. The PIR2 strain expresses the product of the wild-type *pir* gene that is required for replication and maintenance of plasmids containing the R6Kγ origin. In PIR2, plasmids are maintained at approximately 15 copies per cell.

The PIR1 strain contains the mutant *pir-116* allele, which increases the copy number to approximately 250 copies per cell. Both strains are also *endA* to increase the yield of plasmid during isolation, and *recA1* to prevent recombination and rearrangements (see page iv for genotypes). The table below summarizes the application of these strains.

Strain	Application
PIR1	General cloning and maintenance of donor vector constructs
PIR2	For low copy maintenance of donor vector constructs containing toxic genes or libraries.

Continued on next page

Important

It is absolutely essential to transform your donor vector construct into a strain expressing the product of the *pir* gene. **The construct will NOT be maintained or replicated if transformed into a different strain.**

Experimental Outline

The table below outlines the basic steps needed to clone your gene of interest into pUni/V5-His A, B, or C.

Step	Action
1	Determine a strategy to clone your gene of interest into pUni/V5-His A, B, and C. If you wish to include the C-terminal tag on your protein of interest, you must clone in frame with the C-terminal peptide. pUni/V5-His is supplied in three reading frames. Using the diagrams of the multiple cloning site on pages 7-9, determine which version will allow you to clone your gene in frame with the C-terminal peptide.
2	Ligate your gene of interest into the desired version (A, B, or C) of pUni/V5-His.
3	Transform into PIR1 or PIR2 cells.
4	Select transformants on LB plates containing 50 µg/ml kanamycin.
5	Pick transformants and analyze for the presence of your insert. **Note**: We recommend that you sequence your construct to ensure that your gene is in frame with the *loxP* site and the C-terminal peptide, if desired.
6	Isolate plasmid DNA using the method of choice.
7	Proceed to the recombination reaction with the acceptor vector of choice (see the appropriate acceptor vector manual).

Methods

Cloning into pUni/V5-His A, B, and C

Introduction

The Echo™ Cloning System allows you to express your gene of interest in prokaryotic as well as eukaryotic hosts. Careful consideration of the cloning strategy will save you time downstream. Diagrams are provided on pages 7-9 to help you ligate your gene of interest into pUni/V5-His. General considerations for cloning and transformation are discussed below.

General Molecular Biology Techniques

For help with DNA ligations, *E. coli* transformations, restriction enzyme analysis, DNA sequencing, and DNA biochemistry, refer to *Molecular Cloning: a Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

E. coli Strain

The *E. coli* strains, PIR1 and PIR2, contain the *pir* required for replication and maintenance of pUni/V5-His A, B, and C. The donor vector contains the R6Kγ origin and requires the *pir* gene product for replication.

Maintenance of Plasmids

pUni/V5-His A, B, and C contain the kanamycin resistance gene to allow selection of the plasmid using kanamycin. We recommend using the following procedure to propagate and maintain pUni/V5-His A, B, and C:

1. Resuspend each vector in 20 µl sterile water to prepare a 1 µg/µl stock solution. Store the stock solution at -20°C.
2. Use the stock solution to transform a *rec*A, *end*A *E. coli* strain containing the *pir* gene like PIR1 or PIR2.
3. Select transformants on LB plates containing 50 µg/ml kanamycin.
4. Prepare a glycerol stock from a transformant containing plasmid for long term storage (see page 11).

Eukaryotic Expression

For eukaryotic expression, some researchers include a Kozak consensus sequence (Kozak, 1987; Kozak, 1991; Kozak, 1990). Many proteins express well without a Kozak consensus sequence, so inclusion of a Kozak consensus sequence is a matter of personal preference and experience. An example of a Kozak consensus sequence is:

(G/A)NNATG**G**

where the G or A at position -3 and the G at position +4 (shown in bold) are the most critical for function. The ATG initiation codon is shown underlined.

Continued on next page

Cloning into pUni/V5 His A, B, and C, Continued

Cloning Considerations

Refer to the table below for cloning considerations and to the diagrams of the multiple cloning sites on pages 7-9 to assist you.

If you want to…	Then…
recombine into acceptor vectors containing N-terminal tags	clone your gene in frame with the *loxP* sequence. If you are not using acceptor vectors with N-terminal tags, then it is not necessary to clone in frame with the *loxP* sequence.
express your gene of interest in prokaryotes	you may use any one of the ribosome binding sites (RBS) in the vector, if you are cloning your gene such that the initiation codon is within 7-10 base pairs downstream of the RBS. Include a RBS between the *loxP* site and the start codon, if you are using *Bgl* I or any other restriction site after *Bgl* I.
include the C-terminal tag with the V5 epitope and 6xHis tag,	clone your gene in frame with the C-terminal tag. Be sure that your gene does not contain a stop codon.
not include the C-terminal tag,	include the stop codon for your gene of interest or include one in your 3' primer.
secrete your recombinant protein using mammalian, insect, or yeast vectors,	include the appropriate secretion signal (see discussion below) or recombine with an acceptor vector containing a secretion signal sequence.

Secretion Signals

If you are trying to express a protein that is normally secreted from a eukaryote, include the native secretion signal sequence. If you are trying to express and secrete a protein that is **not** normally secreted, you can recombine the donor vector with an acceptor vector containing a secretion signal sequence that is appropriate for the host. **Note:** You should not use an acceptor vector that contains an N-terminal tag (i.e. pcDNA4/ HisMax-E) for recombination and subsequent expression.

If you are trying to express and secrete a protein that is **not** normally secreted in yeast, include the α-factor signal sequence (Brake *et al*., 1984). .

Ligation

Once you have determined a cloning strategy, digest the appropriate version of pUni/V5-His A, B, and C with the selected restriction enzyme. Ligate your gene of interest into pUni/V5-His A, B, or C using standard molecular biology techniques.

Continued on next page

Cloning into pUni/V5 His A, B, and C, Continued

Multiple Cloning Site of pUni/V5-His A

Below is the multiple cloning site for pUni/V5-His A. Restriction sites are labeled to indicate the actual cleavage site. The boxed nucleotides indicate the variable region. Sequencing and functional testing have confirmed the multiple cloning site. **The complete sequence of pUni/V5-His A is available for downloading from our Web site (www.invitrogen.com) or from Technical Service (see page 18).** For a map and a description of the features of pUni/V5-His A, refer to pages 16-17.

pUni Forward priming site

351 GAGCTTAGTA CGTACTATCA ACAGGTTGAA CTGCTGATCA ACAGATCCTC

Kpn I *loxP* site

401 TACGCGGCCG CGGTACC ATA ACT TCG TAT AGC ATA CAT TAT ACG

RBS *Xho* I RBS *EcoR* I *Age* I *Bgl* I

445 AAG TTA TCG GAGGAAT TGGCTCGAGG AATTCACCGG TGCCGTGTGG

BamH I *Apa* I *Aat* II *Stu* I *Pvu* I *Sac* I

491 GCGGATCCGG GCCCGACGTC AGGCCTCGAT CGGAG CTC GGT AAG CCT
Gly Lys Pro

V5 epitope

538 ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT AGC CAT CAT
Ile Pro Asn Pro Leu Leu Gly Leu Asp Ser Ser His His

6xHis tag pUni Reverse priming site

577 CAC CAT CAC CAT TGA AGCTCGCTA TCAGCCTCGA CTGTGCCTTC
His His His His ***

621 TAGTTGCCAG CCATCTGTTG TTTGCCCCTC CCCCGTGCCT

Continued on next page

Cloning into pUni/V5-His A, B, and C, Continued

Multiple Cloning Site of pUni/V5-His B

Below is the multiple cloning site for pUni/V5-His B. Restriction sites are labeled to indicate the actual cleavage site. The boxed nucleotides indicate the variable region. Sequencing and functional testing have confirmed the multiple cloning site. **The complete sequence of pUni/V5-His B is available for downloading from our Web site (www.invitrogen.com) or from Technical Service (see page 18).** For a map and a description of the features of pUni/V5-His B, refer to pages 16-17.

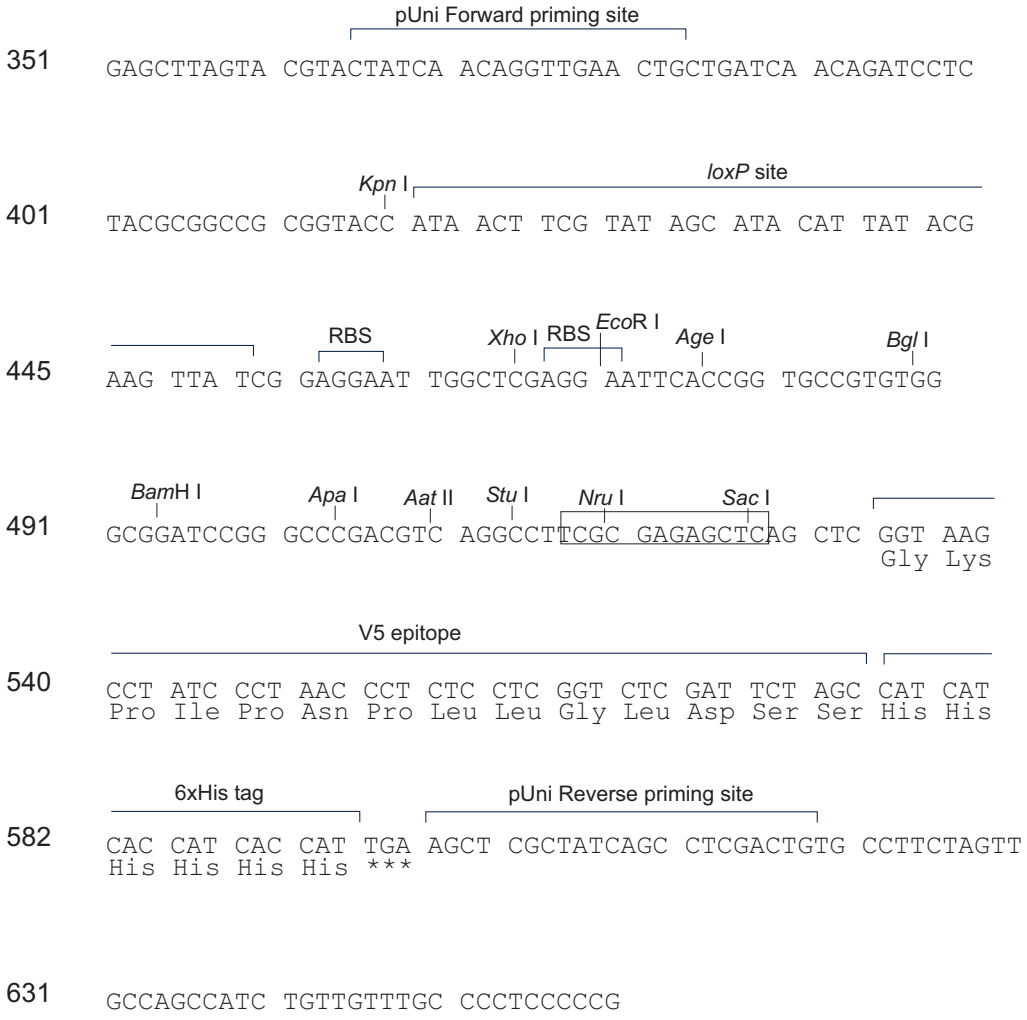
pUni Forward priming site
351 GAGCTTAGTA CGTACTATCA ACAGGTTGAA CTGCTGATCA ACAGATCCTC
Kpn I
loxP site
401 TACGCGGCCG CGGTACC ATA ACT TCG TAT AGC ATA CAT TAT ACG
RBS
Xho I
RBS
EcoR I
Age I
Bgl I
445 AAG TTA TCG GAGGAAT TGGCTCGAGG AATTCACCGG TGCCGTGTGG
BamH I
Apa I
Aat II
Stu I
Sal I
Sac I
491 GCGGATCCGG GCCCGACGTC AGGCCTGTCG ACGAGCTCCC AG CTC
V5 epitope
536 GGT AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT AGC
Gly Lys Pro Ile Pro Asn Pro Leu Leu Gly Leu Asp Ser Ser
6xHis tag
pUni Reverse priming site
578 CAT CAT CAC CAT CAC CAT TGA AG CTCGCTATCA GCCTCGACTG
His His His His His His ***
621 TGCCTTCTAG TTGCCAGCCA TCTGTTGTTT GCCCCTCCCCC

Continued on next page

Cloning into pUni/V5-His A, B, and C, Continued

Multiple Cloning Site of pUni/V5-His C

Below is the multiple cloning site for pUni/V5-His C. Restriction sites are labeled to indicate the actual cleavage site. The boxed nucleotides indicate the variable region. Sequencing and functional testing have confirmed the multiple cloning site. **The complete sequence of pUni/V5-His C is available for downloading from our Web site (www.invitrogen.com) or from Technical Service (see page 18).** For a map and a description of the features of pUni/V5-His C, refer to pages 16-17.



Transformation with One Shot® *E. coli*

Introduction

After ligating the gene of interest into pUni/V5-His A, B, or C, transform the recombinant vector into One Shot® PIR1 or PIR2 *E. coli*.

Before Starting

In addition to general microbiological supplies (i.e. plates, spreaders), you will need the following reagents and equipment.

- 42°C water bath (for heat-shocking chemically competent cells)
- LB plates containing 50 µg/ml kanamycin (two per transformation)
- 37°C shaking and non-shaking incubator

Note

There is no blue-white screening for the presence of inserts. Individual recombinant plasmids need to be analyzed by restriction analysis or sequencing for the presence and orientation of insert. Sequencing primers included in the kit can be used to sequence across an insert in the multiple cloning site to confirm orientation and reading frame.

Preparing for Transformation

For each transformation, you will need one vial of competent *E. coli* and two selective plates.

- Equilibrate a water bath to 42°C.
- Warm the vial of SOC medium to room temperature.
- Warm selective plates at 37°C for 30 minutes.
- Thaw on ice 1 vial of One Shot® *E. coli* cells for each transformation.

One Shot® Transformation Reaction

1. Add 2 µl of the ligation mix into a vial of PIR1 or PIR2 One Shot® cells and mix gently by stirring with a pipette tip. **Do not mix by pipetting up and down**.
2. Incubate on ice for 30 minutes.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer to ice and add 250 µl of room temperature SOC medium.
5. Cap the tube tightly and shake (225 rpm) the tube horizontally at 37°C for 1 hour.
6. Spread 10-50 µl from each transformation onto LB plates containing 50 µg/ml kanamycin plates. **Note**: We recommend that you plate two volumes to ensure that one plate will have well-spaced colonies. For plating small volumes, add 20 µl of SOC to allow even spreading.
7. Incubate overnight at 37°C.
8. Pick ~10 colonies for analysis (see **Analysis of Positive Clones**, next page).

Continued on next page

Transformation with One Shot® *E. coli*, Continued

Analysis of Positive Clones

1. Take the 10 colonies and culture them overnight in LB medium containing 50 µg/ml kanamycin.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01).
3. Analyze the plasmids by restriction analysis to confirm the presence and correct orientation of the insert. Use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert.

Very Few Transformants

If you obtain very few or no transformants with PIR1 cells, it may be that your insert is toxic to *E. coli*. An alternative host, PIR2, is available that contains the wild-type *pir* gene. Expression of the *pir* gene produces wild-type π protein that will maintain copy number at about 15 copies per cell instead of 250 copies.

Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® PIR1 or PIR2 competent cells.

1. Prepare LB plates containing 100 µg/ml ampicillin.
2. Transform with 10 pg of pUC19 per 50 µl of cells using the protocol on page 10.
3. Plate 10 µl of the transformation mix plus 20 µl SOC.

Sequencing

We recommend that you sequence your construct to confirm that your gene is in frame with the C-terminal peptide (if desired). In addition, if you clone in frame with the *loxP* site you will need to sequence to confirm the frame. The pUni Forward and Reverse Sequencing Primers are included to help you sequence your insert. Refer to the diagrams on pages 7-9 for sequence surrounding the multiple cloning site. For the complete sequence of each vector, see our Web site (www.invitrogen.com) or contact Technical Service (page 18).

Long-Term Storage

Once you have identified the correct clone, prepare a glycerol stock for long term storage. We also recommend that you store a stock of your DNA at -20°C or -80°C.

1. Streak the original colony on LB plates containing 50 µg/ml kanamycin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml kanamycin.
3. Grow at 37°C with shaking until the culture reaches stationary phase (OD_{600} = ~1-2).
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

Continued on next page

Recombination with Acceptor Vector

You are now ready to proceed to the recombination reaction. You will need ~100 ng each of your donor vector and the Echo™-adapted acceptor vector for the recombination reaction. Refer to the appropriate acceptor manual for procedures.

Plasmid Preparation

To obtain clean plasmid for recombination and transformation we recommend using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01) or other resin-based DNA purification systems.

Designing Your Own Echo™-Adapted Expression Vector

Introduction

In addition to the already available Echo™-adapted expression vectors you may want to adapt proprietary or specialized vectors for use with the Echo™ Cloning System. The *loxP* or *loxH* recombination sites can be introduced into the expression vector of choice using synthetic oligonucleotides.

loxP or *loxH*

The sequences of the *loxP* and *loxH* sites are shown below. The *loxP* site consists of a 34 bp sequence containing a 13 bp inverted repeat separated by an 8 bp spacer region (Hoess *et al.*, 1982). The inverted repeat (underlined) may form a stem and loop structure that may reduce expression of the gene of interest in some cases. A variation of the *loxP* site (*loxH*) was created to eliminate the formation of a stem and loop structure and potentially improve transcription (Liu *et al.*, 1998). Mutated bases are shown in boldface and underlined.

- *loxP*: ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA T
- *loxH*: AT**T** AC**C** TC**A** TAT AGC ATA CAT TAT ACG AAG TTA T

Note: Data from our experiments do not show any difference in expression levels in vectors containing the *loxP* or the *loxH* site.

Considerations

Consider the following points prior to designing your oligonucleotides.

- Vectors may be constructed using either the *loxP* or the *loxH* site.
- If any vector to be Echo™-adapted contains an N-terminal fusion, then the reading frame noted above should be maintained through the *lox* site.

Construction of Echo™-Adapted Vectors

You can Echo™-adapt your vector at any restriction site that is suitable for your application. You may wish to remove the entire multiple cloning site and N- or C-terminal tags. We recommend that you use two unique restriction sites to facilitate directional cloning. To Echo™-adapt an existing supercoiled vector:

1. Design and order oligonucleotides that contain either the *loxP* or *loxH* site, and the appropriate restriction site overhangs for your vector.
2. Digest your vector with the appropriate restriction enzymes.
3. Anneal the oligonucleotides to create a double stranded fragment.
4. Ligate the annealed oligonucleotide into your vector.
5. Transform competent *E. coli* and select transformants.
6. Isolate recombinant plasmid DNA and sequence to confirm the construction.

An example of Echo™-adaptation of a vector is shown on the next page.

Continued on next page

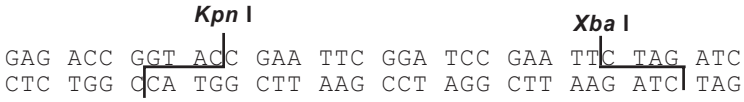
Designing Your Own Echo™-Adapted Expression Vector,
Continued

Example

The example below illustrates how to Echo™-adapt a vector.

Sample Multiple Cloning Site

Kpn I and *Xba* I represent the sites you wish to use to clone in the *loxP* site. Digest the vector with *Kpn* I and X*ba* I.



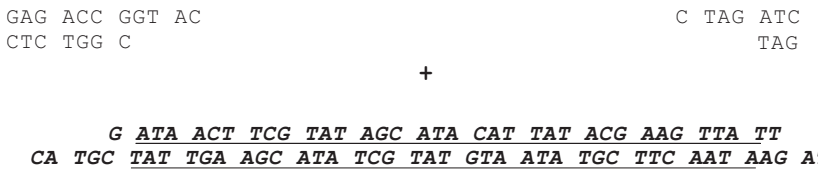
Design Your Oligonucleotides

Design two oligonucleotides such that when they are annealed they create a *loxP* site with ends complimentary to the restriction site overhangs. The *loxP* site is underlined.

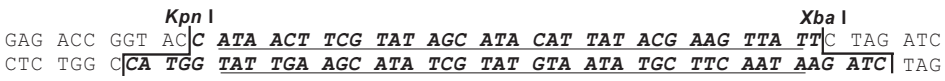
G ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA TT
CA TGC TAT TGA AGC ATA TCG TAT GTA ATA TGC TTC AAT AAG ATC

Create Echo™ (*loxP*)-Adapted Vector

Anneal the two oligonucleotides after they are synthesized. Ligate the annealed oligonucleotide to the digested vector.


GAG ACC GGT AC
C TAG ATC
CTC TGG C
TAG
+
G ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA TT
CA TGC TAT TGA AGC ATA TCG TAT GTA ATA TGC TTC AAT AAG ATC

Echo™-Adapted Vector


Kpn I
Xba I
GAG ACC GGT AC C ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA TT C TAG ATC
CTC TGG C CA TGG TAT TGA AGC ATA TCG TAT GTA ATA TGC TTC AAT AAG ATC TAG

Appendix

Recipes

LB (Luria-Bertani) Medium and Plates

Composition:

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add antibiotic (50 µg/ml kanamycin) if needed.
4. Store at room temperature or at +4°C.

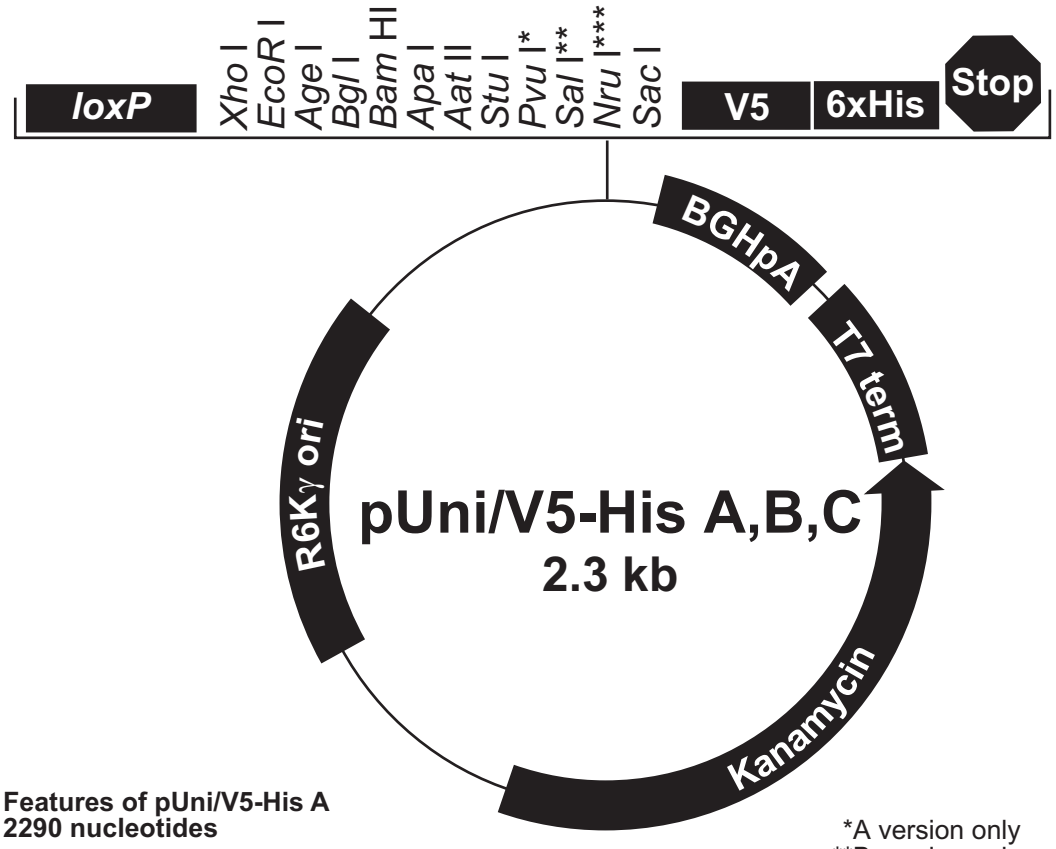
LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, cool to ~55°C, add antibiotic (50 µg/ml kanamycin), and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C, in the dark.

Map of pUni/V5-His A, B, and C Vector

Map

The map below shows the features of pUni/V5-His A, B, and C. For a description of each element, see the next page. For the complete sequence of the vector, you may download it from our Web site (www.invitrogen.com) or call Technical Service (page 18).



Features of pUni/V5-His A
2290 nucleotides

R6K gamma origin: bases 6-397
Uni1 Forward priming site: bases 365-383
loxP site: bases 418-451
Multiple cloning site: bases 464-528
V5 epitope: bases 529-570
6xHis tag: bases 571-588
Uni1 Reverse priming site: bases 592-613
BGH polyadenylation sequence: bases 611-819
T7 transcription termination region: bases 834-962
Kanamycin resistance gene: bases 1141-1935 (C)
Kan promoter: bases 1936-2073 (C)
(C) = complementary strand

*A version only
**B version only
***C version only

Features of pUni/V5-His A, B, and C Vector

Features

The table below describes the features of pUni/V5-His A, B, and C vector.

Feature	Description
loxP	Site-specific recombination site for Cre recombinase (Hoess *et al.*, 1982).
pUni Forward priming site	Permits sequencing of your insert from the 5′ end.
Multiple cloning site	Allows insertion of your gene and facilitates cloning in frame with C-terminal epitope tag.
V5 epitope (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr*)	Permits detection of the recombinant protein after expression by western blot or immunoprecipitation.
6xHis tag	Allows purification of the recombinant protein on metal chelating resin such as Probond™. In addition, the 6xHis-tag can also be used for detection of the recombinant protein using the Anti-His(C-term) antibody.
pUni Reverse priming site	Permits sequencing of your insert from the 3′ end.
Bovine growth hormone polyadenylation sequence	Stabilizes mRNA in eukaryotic cells (Goodwin and Rottman, 1992).
T7 transcription termination region	Stabilizes mRNA in *E. coli*.
Kanamycin resistance gene	Allows selection of transformants in *E. coli*.
R6Kγ origin	Permits replication and maintenance of plasmid in *E. coli* strains containing the *pir* (15 copies of plasmid) or *pir-116* gene (250 copies of plasmid) (see page iv) (Metcalf *et al.*, 1994).

*In pUni/V5-His A, B, and C, the codon for the terminal threonine residue (ACG) was replaced with a codon for a serine residue (AGC). This does not affect subsequent detection using the Anti-V5 antibodies.

Continued on next page

Technical Service

Web Resources

Visit the Invitrogen Web site at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical service contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our Web page (www.invitrogen.com).

Corporate Headquarters:
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008 USA
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail:
tech_service@invitrogen.com

Japanese Headquarters:
Invitrogen Japan
LOOP-X Bldg. 6F
3-9-15, Kaigan
Minato-ku, Tokyo 108-0022
Tel: 81 3 5730 6509
Fax: 81 3 5730 6519
E-mail:
jpinfo@invitrogen.com

European Headquarters:
Invitrogen Ltd
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF, UK
Tel: +44 (0) 141 814 6100
Tech Fax: +44 (0) 141 814 6117
E-mail:
eurotech@invitrogen.com

Material Data Safety Sheets (MSDSs)

MSDSs are available on our Web site at www.invitrogen.com. On the home page, click on **Technical Resources** and follow instructions on the page to download the MSDS for your product.

Limited Warranty

Invitrogen is committed to providing our customers with high-quality goods and services. Our goal is to ensure that every customer is 100% satisfied with our products and our service. If you should have any questions or concerns about an Invitrogen product or service, contact our Technical Service Representatives.

Invitrogen warrants that all of its products will perform according to specifications stated on the certificate of analysis. The company will replace, free of charge, any product that does not meet those specifications. This warranty limits Invitrogen Corporation's liability only to the cost of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. Invitrogen reserves the right to select the method(s) used to analyze a product unless Invitrogen agrees to a specified method in writing prior to acceptance of the order.

Invitrogen makes every effort to ensure the accuracy of its publications, but realizes that the occasional typographical or other error is inevitable. Therefore Invitrogen makes no warranty of any kind regarding the contents of any publications or documentation. If you discover an error in any of our publications, please report it to our Technical Service Representatives.

Invitrogen assumes no responsibility or liability for any special, incidental, indirect or consequential loss or damage whatsoever. The above limited warranty is sole and exclusive. No other warranty is made, whether expressed or implied, including any warranty of merchantability or fitness for a particular purpose.

References

Abremski, K., and Hoess, R. (1984). Bacteriophage P1 Site-Specific Recombination. Purification and Properties of the Cre Recombinase Protein. J. Biol. Chem. *259*, 1509-1514.

Abremski, K., Hoess, R., and Sternberg, N. (1983). Studies on the Properties of P1 Site-Specific Recombination: Evidence for Topologically Unlinked Products Following Recombination. Cell *32*, 1301-1311.

Abremski, K. E., and Hoess, R. H. (1992). Evidence for a Second Conserved Arginine Residue in the Integrase Family of Recombination Proteins. Protein Eng. *5*, 87-91.

Argos, P., Landy, A., Abremski, K., Egan, J. B., Haggard-Ljungquist, E., Hoess, R. H., Kahn, M. L., Kalionis, B., Narayana, S. V. L., Pierson III, L. S., Sternberg, N., and Leong, J. M. (1986). The Integrase Family of Site-Specific Recombinases: Regional Similarities and Global Diversity. EMBO J. *5*, 433-440.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994). Current Protocols in Molecular Biology (New York: Greene Publishing Associates and Wiley-Interscience).

Brake, A. J., Merryweather, J. P., Coit, D. G., Heberlein, U. A., Masiarz, G. R., Mullenbach, G. T., Urdea, M. S., Valenzuela, P., and Barr, P. J. (1984). a-Factor-Directed Synthesis and Secretion of Mature Foreign Proteins in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA *81*, 4642-4646.

Goodwin, E. C., and Rottman, F. M. (1992). The 3´-Flanking Sequence of the Bovine Growth Hormone Gene Contains Novel Elements Required for Efficient and Accurate Polyadenylation. J. Biol. Chem. *267*, 16330-16334.

Hoess, R. H., Ziese, M., and Sternberg, N. (1982). P1 Site-Specific Recombination: Nucleotide Sequence of the Recombining Sites. Proc. Natl. Acad. Sci USA *79*, 3398-3402.

Kozak, M. (1987). An Analysis of 5´-Noncoding Sequences from 699 Vertebrate Messenger RNAs. Nucleic Acids Res. *15*, 8125-8148.

Kozak, M. (1991). An Analysis of Vertebrate mRNA Sequences: Intimations of Translational Control. J. Cell Biology *115*, 887-903.

Kozak, M. (1990). Downstream Secondary Structure Facilitates Recognition of Initiator Codons by Eukaryotic Ribosomes. Proc. Natl. Acad. Sci. USA *87*, 8301-8305.

Liu, Q., Li, M. Z., Leibham, D., Cortez, D., and Elledge, S. (1998). The Univector Plasmid-Fusion System, a Method for Rapid construction of Recombinant DNA Without Restriction Enzymes. Current Biology *8*, 1300-1309.

Liu, Q., Li, M. Z., Liu, D., and Elledge, S. J. (1999). Rapid Construction of Recombinant DNA by the Univector Plasmid-Fusion System. Methods in Enzymology, in press.

Metcalf, W. W., Jiang, W., and Wanner, B. L. (1994). Use of the *rep* Technique for Allele Replacement to Construct New Escherichia coli Hosts for Maintenance of R6K Gamma Origin Plasmids at Different Copy Numbers. Gene *138*, 1-7.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Plainview, New York: Cold Spring Harbor Laboratory Press).

Sternberg, N., Hamilton, D., Austin, S., Yarmolinsky, M., and Hoess, R. (1981). Site-Specific Recombination and its Role in the Life Cycle of P1. CSH Symp. Quant. Biol. *45*, 297-309.

©1999-2006, 2010 Invitrogen Corporation. All rights reserved.

For research use only. Not intended for any animal or human therapeutic or diagnostic use.

invitrogen™

Corporate Headquarters
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008
T: 1 760 603 7200
F: 1 760 602 6500
E: tech.service@invitrogen.com

For country-specific contact information visit our web site at **www.invitrogen.com**